Constant expression of hexose-6-phosphate dehydrogenase during differentiation of human adipose-derived mesenchymal stem cells

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Abstract

The reductase activity of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) plays an important role in the growth and differentiation of adipose tissue via the prereceptorial activation of glucocorticoids. This enzyme colocalizes with hexose-6-phosphate dehydrogenase (H6PD) at the luminal surface of the endoplasmic reticulum membrane, and the latter enzyme provides NADPH to the former, which can thus act as an 11β-reductase. It was suggested that, during adipogenesis, the increased expression of H6PD causes a dehydrogenase-to-reductase switch in the activity of HSD11B1. However, only the expression of the HSD11B1 has been extensively studied, and little is known about the expression of H6PD. Here, we investigated the expression and the activity of H6PD in the course of the differentiation of human adipose-derived mesenchymal stem cells (ADMSCs) and murine 3T3-L1 cells. It was found that H6PD is already present in adipose-derived stem cells and in 3T3-L1 fibroblasts even before the induction of adipogenesis. Moreover, mRNA and protein levels, as well as the microsomal H6PD activities remained unchanged during the differentiation. At the same time a great induction of HSD11B1 was observed in both cell types. The observed constant expression of H6PD suggests that HSD11B1 acts as a reductase throughout the adipogenesis process in human ADMSCs and murine 3T3-L1 cells.

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Introduction

Hexose-6-phosphate dehydrogenase (H6PD) catalyzes the first two reactions of the pentose phosphate pathway in the endoplasmic reticulum (ER), thereby generating reduced NADPH (NADP±) within the luminal compartment (Buetler & Morrison 1967, Takahashi & Hori 1978, Ozols 1993, Collard et al. 1999, Mason et al. 1999, Clarke & Mason 2003). It uses a separate pool of glucose-6-phosphate and NADP± distinct from the cytosolic ones (Bánhegyi et al. 2004, Czegle et al. 2005). It has been proposed that the luminal [NADPH]/[NADP±] ratio determines the direction of the activity of the ER luminal enzyme 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1; Atanasov et al. 2004, Hewitt et al. 2004, Odermatt et al. 2006). Indeed, HSD11B1 in vitro catalyzes the reversible conversion between (inactive) 11-ketoglucocorticoids and (active) 11-hydroxylglucocorticoids, although in intact cells acts predominately as a reductase consuming NADPH and activating glucocorticoids.

The local activation of glucocorticoids could have various pathophysiological roles, such as stimulation of adipogenesis (Seckl & Walker 2004, Tomlinson et al. 2004, Draper & Stewart 2005). An enhanced lipogenesis, for example, is considered to be key event in abdominal obesity and metabolic syndrome (Tomlinson & Stewart 2002, Seckl et al. 2004, Andrew et al. 2005).

An important role in adipogenesis has been attributed to H6PD (Atanasov et al. 2004, Hewitt et al. 2004, Odermatt et al. 2006). It was demonstrated that mutations in genes of both HSD11B1 and H6PD in a triallelic digenic model of inheritance result in the loss of HSD11B1 reductase activity (Draper et al. 2003). During the differentiation of human omental adipose stromal cells, a switch was observed in HSD11B1 activity from dehydrogenase to reductase direction (Bujalska et al. 2002). Later an increased H6PD expression was reported during adipocyte differentiation and it was associated with the previously described switch in HSD11B1 activity (Atanasov et al. 2004). A transient mild increase in...
**H6PD** mRNA levels was observed during the differentiation of the transformed human s.c. preadipocyte cell line Chub-S7 (Bujalska et al. 2008). These results were supported by the observation that HSD11B1 has increased reductase and decreased dehydrogenase activity when coexpressed with H6PD in HEK-293 cells (Atanasov et al. 2004, Bujalska et al. 2005, Nashev et al. 2007). Transfection of HEK-293 cells with an H6PD siRNA decreased HSD11B1 reductase activity (Bujalska et al. 2005). Moreover, it was found that in H6PD knockout mice, the HSD11B1-mediated prereceptorial glucocorticoid activation was impaired (Lavery et al. 2006). Therefore, it was postulated that the early induction of H6PD was responsible for the appearance of HSD11B1 reductase activity and the consequent prereceptorial glucocorticoid activation in preadipocyte differentiation.

However, the theory has some drawbacks. The absence or low activity of a luminal NADPH-generating enzyme is not enough to postulate a low [NADPH]/[NADP+] ratio. One should suppose the presence of an NADPH-dependent reductase in the same compartment that could regenerate NADP+ for the dehydrogenase activity of HSD11B1; however, such activity has not been reported in preadipocytes. On the other hand, several reports showed that H6PD is present in a variety of cell types, including fibroblast (Takahashi & Hori 1978). Moreover, NADPH-generating enzymes other than H6PD can be found in the ER lumen (Margittai & Bánhegyi 2008). These circumstances can query the postulated role of H6PD in adipogenesis. Therefore, we (re)examined the expression and activity of H6PD and HSD11B1 during the process of adipogenesis. To this end, we used highly undifferentiated cells prepared from human adipose tissue, which present with the differentiation properties of mesenchymal stem cell – referred to here as adipose-derived mesenchymal stem cells (ADMSCs) – and murine 3T3-L1 fibroblasts.

As a main result, we observed that H6PD expression and activity do not change during adipogenesis, while HSD11B1 expression largely increases in our experimental systems. As a main conclusion, therefore, the hypothesis that the appearance of H6PD confers the reductase activity to HSD11B1 does not have a general validity.

**Materials and methods**

**Materials**

Cortisone, cortisol, dexamethasone, isobutylmethyl xanthine (IBMX), glucose-6-phosphate, NADP+, NADPH, triton X-100, collagenase (type IA), and 4-morpholineproanesulfonic acid (MOPS) were purchased from Sigma Chemical Co. Dulbecco’s minimum essential medium (DMEM), α-Minimum Essential Medium Eagle (α-MEM), and fetal bovine serum (FBS) were from Cambrex. All other reagents and solvents were of analytical grade.

**Preparation, culturing, and differentiation of ADMSCs**

ADMSCs were prepared from human adipose tissue obtained from patients undergoing elective liposuction or lipectomy procedures. Cells were isolated using a protocol previously described (Zuk et al. 2001), with some modifications. Adipose tissue was minced into small pieces and washed with PBS, containing antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin). To isolate the stromal-vascular fraction (SVF), the tissue fragments were treated with 0.075% collagenase and 0.25 mM CaCl₂, in PBS (4 ml of solution for g of tissue), for 1 h, at 37 °C, under gentle agitation. Digestion was stopped by adding an equal volume of α-MEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine and the cell suspension was centrifuged at 600 g, for 10 min, at 22 °C. The pellet was resuspended in culture medium, filtered through nylon sheet (100 µm mesh), and centrifuged again as above. The SVF pellet was resuspended in culture medium and filtered again through 70 µm nylon mesh. The stromal cells were counted and plated at 1 00 000 cells/cm² onto tissue culture plastic dishes for 24 h at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Contaminating erythrocytes and non-adherent cells were then removed by washing dishes with PBS. Cells were cultured for 7–10 days to reach a 70–80% confluence, detached with 0.25% Trypsin/0.53 mM EDTA in PBS, and seeded at 4000 cells/cm² and subcultured after 7 days. Cells were used between passages 2–5. For differentiation into adipocytes, cells were plated at 20 000 cells/cm² in MesenCult Basal Medium supplemented with 10% FBS, antibiotic, and 1% l-glutamine. After 24–48 h, the same medium containing 1 µM dexamethasone, 10 µM insulin, 0.5 mM IBMX, and 200 µM indomethacin, was added. After 21 days of adipogenic stimulation, cells were fixed in 4% PFA for 1 h and incubated with Oil Red-O to stain lipid vacuoles. Chondrogenic and osteogenic differentiations were induced as described elsewhere (Gallo et al. 2007).

**Culturing and differentiation of 3T3-L1 fibroblasts**

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD, USA) were cultured and differentiated as described by Frost & Lane (1985). Briefly, 2 days after confluence – referred to as 0 day – adipogenesis was induced by the addition of DMEM containing 10% FBS, 5 µg/ml insulin, 0.5 mM IBMX, and 0.5 µM dexamethasone. After 2 days, the medium was removed and cells were further cultured for 2 days in DMEM containing...
10% FBS and 5 μg/ml insulin. Cells were then maintained in DMEM containing 10% FBS until use, normally at the seventh day after starting adipogenesis.

Real-time RT-PCR assay

Total RNA from ADMSCs and 3T3-L1 cells was isolated with RNeasy Plus Mini kit (Qiagen), according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed in a final volume of 20 μl, using the SuperScript first-strand synthesis System III (Invitrogen) and oligo(dT)12–18.

Expression levels of HSD11B1 and H6PD – as well as of Rplp0 and β-2-microglobulin (B2M) as reference genes – were quantified by fluorescent real-time PCR with an Opticon Monitor 3 (Biorad, Segrate, Milan, Italy). Analyses were performed in triplicate in a 25 μl reaction mixture. cDNA (1 μl) was amplified with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 50 nM of the sense and antisense primers. Amplification protocol was: 95°C (15 min), 45 cycles of 95°C (20 s), variable (20 s), 72°C (20 s). Sequences of PCR primers for human and murine genes are given in Table 1. The annealing temperature and the amplification efficiency for each primer pair are also indicated. Amplification efficiency was evaluated by serial (tenfold) dilutions of suitable template cDNA and was calculated as reported (Pfaffl 2001). Every assay was run in triplicate and supernatants were used for western blot analysis.

Preparation of lysates from ADMSCs

ADMSCs (before and after the induction of adipogenesis) were washed with PBS, scraped off, and incubated in 20 mM HEPES, 1 mM EDTA (pH 7.4), including protease inhibitors (Complete, Roche). Homogenates were centrifuged at 10 000 g for 20 min, and the supernatant was subsequently centrifuged at 100 000 g for 120 min. The microsomal pellet was resuspended in the Mops buffer and stored in liquid nitrogen until use. Human liver and omental adipose tissue samples were small portions of wedge samples obtained for the investigation of the original condition for which the patient was referred. The local Ethical Committee of the Semmelweis University approved this study.

Western blot analysis

Microsomal proteins or proteins of cellular lysates were resolved on 12% polyacrylamide gels and blotted on nitrocellulose. Immunoblots were probed with the different antibodies and analyzed by enhanced chemiluminescence (ECL). Horseradish peroxidase-conjugated anti-rabbit IgG-specific secondary antibody and an ECL kit were from Amersham Biosciences. The H6PD protein was immunoreacted with a rabbit polyclonal antiserum against the lactonase domain (residues 539–791) of human H6PD (Marcolongo et al. 2007) kindly

Table 1 Primer sequences for real-time RT-PCR. Sense and antisense primers are given for each gene at 5′–3′ direction as specified. The annealing temperature and the amplification efficiency for each primer pair are also indicated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5′–3′)</th>
<th>Antisense (5′–3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplification efficiency</th>
</tr>
</thead>
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<tr>
<td>mRplp0</td>
<td>AGG CGC GTC CTG CTA TTG TCT</td>
<td>CCG CAG GGG CAG CAG TGG T</td>
<td>58</td>
<td>1.99</td>
</tr>
<tr>
<td>mHsd11b</td>
<td>CCA GCA AAG GGA TTG GAA GAG A</td>
<td>GTA GTG AGC AGA GGA TGC TCC</td>
<td>55.2</td>
<td>1.93</td>
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<tr>
<td>mH6pd</td>
<td>GGA GCT GAT CTC CAA GGT GCC</td>
<td>CCC TGA CAG TGC CAG GGT GAA</td>
<td>56</td>
<td>1.77</td>
</tr>
<tr>
<td>hB2M</td>
<td>TGCCTGCTCCATGTGGAATGATATCT</td>
<td>TCTCTGCTCCCACACTCTAAGT</td>
<td>53</td>
<td>1.92</td>
</tr>
<tr>
<td>hHSD11b</td>
<td>CCGCAAGGAAATCGGAGAGAG</td>
<td>GTAGTGTGTAGGCGTGTCCT</td>
<td>55.4</td>
<td>1.85</td>
</tr>
<tr>
<td>hH6PD</td>
<td>ATGAAAAAGGACCCTGGATGCTGAA</td>
<td>CTCCTAGGCCACAGGGGTAG</td>
<td>57</td>
<td>1.86</td>
</tr>
</tbody>
</table>

m, murine; h, human.

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provided by Dr Evan Schaftingen (Laboratoire de Chimie Physiologique, ICP and UCL, Brussels B-1200, Belgium). The HSD11B1 protein was immunorevealed with rabbit polyclonal antibodies recognizing a 15 amino acid sequence common to the human and murine proteins (Cayman Chemical, Ann Arbor, MI, USA).

**Measurement of microsomal H6PD activity**

Just before each experiment, 3T3-L1 microsomes were thawed and washed by rapid centrifugation, and the microsomal pellets resuspended in Mops buffer (Marcolongo et al. 2007). H6PD activity was evaluated in the microsomal suspensions (0.1–0.33 mg of protein per ml, in the Mops buffer) by measuring NADPH formation upon the subsequent addition of 2 mM NADP$^+$, 1 mM G6P, and Triton X100 (1% final concentration) as previously reported in details (Marcolongo et al. 2007). Fluorescence of NADPH was monitored at a 350 nm excitation and a 460 nm emission wavelength using a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA).

**HSD11B1 activities in ADMSCs and 3T3-L1 cells**

Reduction of cortisone to cortisol and oxidation of cortisol to cortisone were measured by incubating the cells (0.2–1×10$^6$ in a final volume of 1 ml) in the presence of 1 µM steroid, for 2 h at 37 °C. The reaction was terminated by the addition of equal volume of ice-cold methanol and the samples were kept at −20 °C until analysis. After the sedimentation of the precipitates by centrifugation (20 000 g for 10 min at 4 °C), the cortisol and cortisone contents of the supernatants were measured by HPLC (Waters Alliance 2690) using a Nucleosil 100 C18 column (5 µm 25×0.46) (Teknokroma. Barcelona, Spain). The mobile phase was isocratic methanol–water (58:42, v/v) at 0.7 ml/min flow rate and absorbance was detected at 245 nm wavelength (Waters Dual λ Absorbance Detector 2487). The retention times of cortisone (14:1 min) and cortisol (16:8 min) were determined by injecting standards.

**Results**

**Characterization of the human ADMSCs**

Previous studies on glucocorticoid-induced adipogenesis have been conducted in rodent cell lines such as the mouse 3T3-L1 or in SVF from human adipose tissue. However, SVF represents a heterogeneous cell population (Varma et al. 2007, De Sousa Peixoto et al. 2008); therefore, we prepared mesenchymal precursor/stem cells from human adipose tissue ADMSCs, as these cells represent undifferentiated precursor of several mesenchymal lineages including chondrocytes, osteocytes, and adipocytes (Zuk et al. 2001, 2002). The differentiative potentials of these mesenchymal cells were confirmed following culture in specific media for differentiation in adipogenic, osteogenic, and chondrogenic phenotypes as detailed in the ‘Materials and methods’ section. The degree of adipogenic, osteogenic, and chondrogenic differentiations was evaluated by specific staining with Oil Red-O, Alizarin Red S, and Alcian blue, as shown in Fig. 1. Differentiation of these cells in the three lineages was also confirmed by RT-PCR analysis of specific genes (peroxisome proliferator-activated receptor-γ, alkaline phosphatase, and decorin respectively; data not shown).

**Cortisone reductase activity increases in ADMSCs and 3T3-L1 cells during differentiation**

Undifferentiated ADMSCs reduced a negligible amount of cortisone to cortisol. The adipogenic differentiation was associated with a remarkable increase in the reductase activity: at the 7th and 14th days of the adipogenesis-inducing treatment, ADMSCs converted 36.5 and 32.8% (means of two separate experiments) of the added cortisone to cortisol respectively.

In accordance with the previous observations (Napolitano et al. 1998), a little (lower than 2%) cortisone to cortisol conversion was measured in undifferentiated 3-T3-L1 cells. Differentiation was accompanied by a several-fold elevation of the reductase activity, reaching a peak value at the seventh day (Fig. 2). Dehydrogenase activity (i.e., cortisol to cortisone conversion) was under the detection limit in the undifferentiated cells, and remained very low even at the seventh day (approx 3%, see Fig. 2).

**Increased reductase activity is due to HSD11B1 rather than H6PD induction**

Increased 11-ketoglucocorticoid reductase activity upon adipogenesis has been attributed to both HSD11B1 and H6PD induction. However, the induction of H6PD was demonstrated only at mRNA level. Our immunoblot analysis using an antibody against the lactonase domain of H6PD (Marcolongo et al. 2007) revealed that the amount of this protein in the undifferentiated ADMSCs and 3T3-L1 fibroblasts is well comparable with that observed after the induction of adipogenesis in both cell types (Fig. 3). By contrast, the HSD11B1 protein was hardly detectable in ADMSCs and 3T3-L1 fibroblasts, but a strong induction could be demonstrated during the differentiation process (Fig. 3).

Since the amount of ADMSCs did not allow the preparation of sufficient amounts of microsomal fraction – and cell lysates cannot be used for obvious reasons – H6PD activity was measured in microsomes prepared from 3T3-L1 cells. In accordance with the results obtained by immunoblotting, the H6PD activity was practically
constant during the differentiation (day 0 = 2.74 ± 0.91; day 3 = 2.99 ± 1.54; day 7 = 2.92 ± 1.14 nmol/min per mg microsomal protein, n = 3, means ± S.D.).

**H6PD mRNA levels do not change during adipogenesis**

To further confirm the results obtained at protein level, *H6PD* and *HSD11B1* mRNA levels were measured by real-time RT-PCR in ADMSCs and 3T3-L1 fibroblasts before and after the induction of adipogenesis. While the relative expression of *HSD11B1* was strongly elevated in both cell types in fully differentiated cells (37-fold in ADMSCs and 204-fold in 3T3-L1 cells, Fig. 4A and B respectively), *H6PD* expression remained at the same level (Fig. 4).

**Discussion**

The present results demonstrate that H6PD mRNA, protein, and activity are present in undifferentiated cells including mesenchymal precursor/stem cells ADMSCs and 3T3-L1 fibroblasts even before the induction of adipogenesis. Moreover, the mRNA and protein levels, as well as the microsomal H6PD activities remain more or less unchanged during the differentiation toward an adipocyte phenotype. These findings are in line with the supposed housekeeping function of the enzyme. In fact, a systematic screening of the occurrence of H6PD protein in various tissues showed that it is a ubiquitous enzyme (Banhegyi, unpublished results; see also Gomez-Sanchez et al. 2007).

The presence of an active H6PD protein in progenitor cells suggests that HSD11B1 must act as a reductase throughout adipogenesis. A very recent study supports this view demonstrating that the mouse adipose stromal vascular cells (ASVCs) express H6PD, consistent with dominant reductase activity of HSD11B1 (De Sousa-Peixoto et al. 2008). Conflicting results were previously reported by others in human ASVCs showing a dehydrogenase-to-reductase switch during the adipogenic differentiation (Bujalska et al. 2005, Nashev et al. 2007).
However, the initial dehydrogenase activity observed in the ASVCs might be due to an HSD11B1-independent cortisol dehydrogenase activity. ASVCs are a heterogeneous population including preadipocytes, fibrocytes, monocytes, and endothelial cells. Interestingly, HSD11B1 is expressed in preadipocytes only, at levels comparable with differentiated adipocytes (De Sousa Peixoto et al. 2008). The cellular heterogeneity of the preparation might thus explain the relatively high dehydrogenase activity before adipogenic differentiation. For example, endothelial cells – potentially present in the preparation – express 11β-HSD2 (Brem et al. 1998). As far as the reconstitution experiments (Atanasov et al. 2004, Bujalska et al. 2005) are concerned, attention should be posed on the level of (over)expression of HSD11B1. In the presence of non-physiological, high level of the enzyme, the regeneration of NADPH by H6PD might not be sufficient to allow the reductase activity. Alternatively, the overexpressed HSD11B1 might not be properly targeted to the inner face of the ER membrane but directly or indirectly – in ER-derived bodies resulting from an ER overloading/stress – to the cytosol, where it works as a dehydrogenase (Odermatt et al. 1999). A study in 3T3-L1 cells, whose HSD11B1 has been silenced by short hairpin RNA constructs (Kim et al. 2007), is in line with the aforementioned hypothesis. These authors observed that the re-expression of HSD11B1 at relatively low levels restores the dehydrocorticosterone-induced differentiation, a process that requires the HSD11B1-dependent reductase activity. Paradoxically, high levels of re-expression of the HSD11B1 enzyme resulted in no dehydrocorticosterone dependent differentiation, indicating a lack of reductase activity of the overexpressed enzyme. A further theoretical reasoning is that a

**Figure 2** Reduction of cortisone to cortisol and oxidation of cortisol to cortisone by 3T3-L1 cells in the course of adipogenesis. Adipogenic differentiation was induced in 3T3L1 cells and the conversion of cortisone to cortisol or cortisol to cortisone was measured at the indicated days of differentiation. Reduction of cortisone to cortisol and oxidation of cortisol to cortisone were measured by incubating the cells (0–2–1×10⁶ in a final volume of 1 ml) in the presence of 1 μM steroid, for 2 h at 37 °C. Cortisol and cortisone contents of the samples were measured by HPLC. The activities are reported as % of conversion of the added steroid and values are means ± s.d. of four independent experiments.

**Figure 3** Expression of H6PD and HSD11B1 proteins in ADMSC and 3T3-L1 cells before and after the induction of adipogenesis. Lysates were prepared from ADMSCs before (0 day) and at 7 and 14 days after initiating the treatments to induce adipogenesis. As controls, microsomes were prepared from liver as well as human adipose tissue specimens. Microsomes were prepared from 3T3-L1 fibroblasts before (0 day) and at 3 and 7 days after initiating the treatments to induce adipogenesis. Proteins of cell lysates as well as of microsomal fractions were separated by 12% SDS-PAGE. Gels were blotted on a nitrocellulose membrane, and the H6PD and HSD11B1 proteins were immunorevealed with antibodies toward the lactonase domain of the H6PD protein or with rabbit polyclonal antibodies, recognizing a 15 amino acid sequence common to the human and murine protein as an epitope of the HSD11B1 protein. In the case of ADMSC cell lysates, 20 μg protein were applied; a representative result of three experiments is shown. In the case of liver and adipose tissue microsomes, 30 μg protein of two different preparations were applied. In the case of microsomes from 3T3-L1 fibroblasts, 40 μg of protein were applied; a representative result of four experiments is shown. On the right side of the immunoblots, the size of molecular mass markers is shown in kDa units.
NADP⁺-regenerating system should be present in the ER lumen in order to permit a continuous HSD11B1 dehydrogenase activity, but such enzyme systems have never been described.

Our results confirm that H6PD plays an important role in the prereceptorial glucocorticoid activation in adipogenesis, assisting HSD11B1 in its reductase activity. During the adipogenic differentiation, in the continuous presence of H6PD activity, the dramatic induction of HSD11B1 per se can explain the crescendo of reductase activity.

In terms of human disease, our results endorse the strategy for a targeted inhibition of HSD11B1 in patients with obesity and metabolic syndrome, as this enzyme appears always to contribute in local glucocorticoid activation (Tomlinson & Stewart 2005). Theoretically, H6PD can also be a reasonable therapeutic target. However, the fact that H6PD is constantly expressed, thus likely being a housekeeping enzyme, raises some concerns. H6PD is likely a main source of reduced pyridine nucleotides in the luminal environment (Piccirella et al. 2006), which – in addition to the cofactor supply for HSD11B1 – may serve to (vital) cell functions as well. This view is supported by the observation that the indirect blockade of H6PD – through the downregulation of the ER glucose-6-phosphate transporter – induces apoptosis in neutrophils (Leuzzi et al. 2003) and glioma cells (Belkaid et al. 2006a,b). Moreover, it has been recently reported (Lavery et al. 2008) that H6PD knockout mice develop a skeletal myopathy associated with the unfolded protein response, which does not seem to be related to local glucocorticoid inactivation or insufficient activation. Further studies are required to clarify the underlying mechanisms.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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